such components. Support for this amendment is found in the specification at, for example, page 4, lines 1-3.

Claim 2 has been amended to recite that the mobile phase further includes a modifier. Support for this amendment is found in original claim 1, and in the specification at, for example, page 4, lines 9-23. (*Id.*)

It is submitted that no new matter has been introduced by the foregoing amendments. Approval and entry of the amendments is respectfully solicited.

Claims 1-8 were rejected solely under 35 USC §103(a) as being unpatentable over Zhang *et al.*, (CA 113:198104, abstract of Zhongguo Yiyao Gongye Zazhi (1990), 21(6), 256-61) ("Zhang") in view of Lee *et al*, (CA 124:105153, abstract of J. Microcolumn Sep. (1995), 7(5):477-83) ("Lee"). (Paper No. 6 at 2).

For the reasons set forth below the rejection, respectfully is traversed.

Zhang discloses "HPLC determination of vitamin D_3 preparation." (Title). The Abstract relied on by the rejection recites the following:

> In the column system suitability test following irradn. of heated vitamin D_3 soln. by UV with main wave length 254 and 365 nm for 5 min, 6 isomers were sepd. with the normal-phase HPLC (Waters Resolve Silica column, 0.3% n-pentanol in hexane as mobile phase, detected at 254 nm), with resoln. factor, R, >1.0. The linearity was obtained in 0.5-60 .mu.g vitamin D_3. The low concn. (1ppm) prepn. could be detd. by internal (di-Me phthalate) method or external (thermal equil.) method with error 10%. The error in detn. of high-concn. prepn. was 3%.

(Abstract).

Lee discloses the use of an "enhanced liquidity" or a low viscosity liquid mobile phase as an eluent in reversed phase HPLC. (Page 477, Col. 1). In Lee, "***all***" experiments disclosed were performed using a 0.7/0.30 mole fraction methanol/H_2O mixture or a

0.49/0.21/0.30 mole fraction methanol/H_2O/CO_2 mixture. (Page 478, Col. 1). Lee further discloses that "***[r]eversed phase HPLC, unlike normal phase HPLC and SFC*** (supercritical fluid chromatography), ***is the only technique by which vitamins D_2 ... and D_3 ... can be resolved***. (Page 482, Col. 2). In Figure 5, Lee presents a chromatogram of fat soluble vitamins, including vitamin D_3, and concludes that the separation "is attributed to the ... ***reversed phase conditions***." (*Id.*).

In making the rejection, the Examiner asserted that Zhang "teaches the separation of 6 isomers separated by using irradiation technique [sic] and the purification on silica column (stationary phase)." (Paper No. 6 at 3). The Examiner acknowledged, however, that the "[I]nstant claims differ from the reference in claiming the liquid CO_2 for separation by column chromatography using liquid CO_2." (*Id.*). To fill the acknowledged gap, the Examiner relied upon Lee as teaching "the separation of coal tar vitamins and other related compounds" using an "enhance[d] fluidity liquid mobile phase containing CO_2/methanol/water" in a column. (*Id.*).

The Examiner then asserted that the "references are combinable because they are from the same field of endeavor." (*Id.*). The Examiner then contended that "it would have been obvious to one skilled in the art to combine the teachings of prior art supra to separate the vitamin D derivatives particularly when Zhang *et al.* teaches irradiation technique and the purification on silica column (stationary phase) and Lee *et al.* teaches the use of liquid CO_2 for separation." (*Id.* at 3-4). The Examiner further contended that "***ample motivation***" for separating vitamin D_3 as claimed is found in the prior art, and that there was "***nothing unobvious***" about the process for separating the vitamin D_3 as claimed. (*Id.* at 4).

Initially and with all due respect, the rejection uses the wrong standard for determining obviousness. The rejection relies upon "***ample motivation***" (Paper No. 6 at 4),

"***nothing unobvious***" (*Id.*) and "***reasonably infer***" (*Id.* at 5) standards that are not found in the statute or precedential authority. Reliance on such novel standards for a §103(a) rejection, *e.g.* that there is "nothing unobvious" recited in the claim is simply improper. It is the Examiner's burden to set forth evidence sufficient to support a case of *prima facie* obviousness - not to make unsupported conclusions that there is "nothing unobvious" about the claim.

Similarly, whether or not "ample motivation" is found in the prior art to combine references, it is also the Examiner's burden to explain with particularity where the motivation to combine ***and*** where the reasonable expectation of success are found. As is fundamental, a *prima facie* case of obviousness must be based on facts, "cold hard facts." *In re Freed*, 165 USPQ 570, 571-72 (C.C.P.A. 1970). When the rejection is not supported by facts, it cannot stand. *Ex parte Saceman*, 27 USPQ2d 1472, 1474 (B.P.A.I. 1993). A mere invocation of the "prior art" without reference to the particular document(s) relied on is insufficient to support a conclusion of obviousness under §103(a).

As is well settled, an Examiner cannot establish obviousness by locating references which describe various aspects of a patent applicant's invention without also providing evidence of the motivating force which would ***impel*** one skilled in the art to do what the patent applicant has done. *Ex parte Levengood*, 28 USPQ2d 1300, 1301-02 (BPAI 1993). "Reasonably infer," just like "ample motivation" and "nothing unobvious," is simply not the standard on which conclusions of obviousness may be based. In sum, the rejection fails to provide any reason ***why*** one would be motivated, let alone impelled, to combine the references in the manner suggested by the Examiner. In short, the rejection fails to meet its burden because it relies on the wrong legal standards. And, thus it should be withdrawn.

Even if the rejection is deemed to be founded on the correct legal standard, it relies on Zhang, which is an abstract that is so factually incomplete that it cannot be used in the manner suggested by the Examiner. For example, Zhang recites a "column suitability test." The rejection, however fails to provide any explanation of how a ***column suitability test*** relates to a ***process for isolating*** vitamin D_3 or previtamin D_3 as claimed.

Moreover, Zhang states that 6 isomers were separated ("sepd.") with normal phase HPLC. The rejection assumes, without a shred of evidence, that the "6 isomers" recited in Zhang refer to isomers of vitamin D_3 or previtamin D_3 as recited in claim 1.

Zhang concludes by presenting percent error in ***detection*** ("detd.") data of some substance (whether a specific compound or mixture of 6 isomers is not clear) in a high concentration preparation (3% error) and a low concentration preparation (10% error). The rejection, however, fails to explain how a method of ***detection*** relates in any way to the presently claimed process for ***isolating*** vitamin D_3 or previtamin D_3.

In sum, the rejection reads into Zhang a number of elements recited by the present claims without providing any evidence or reasoning for doing so. In the absence of such evidence, it can only be concluded that the present specification was used as the template to fill in the factual gaps noted above with respect to Zhang. But this type of hindsight reconstruction is expressly prohibited. *In re Dow Chem. Co.*, 5 USPQ2d 1529, 1531-1532 (Fed. Cir. 1988) ("There ***must*** be a reason or suggestion in the prior art for selecting the procedure used, other than the knowledge learned from the applicant's disclosure."). For this additional reason, the rejection should be withdrawn.

Moreover, in attempting to rebut the arguments presented in the Response mailed March 14, 2000, the Examiner asserted that "a rejection is good not only for what it teaches by

direct anticipation but also for what one of ordinary skill might reasonably infer from the teaching." (Paper No. 6 at 5). Thus, in the Examiner's own words, the linchpin of the rejection is not what is disclosed in the Zhang and Lee documents, but what one skilled in this art would "infer" from them. In essence, the rejection argues that both the motivation to combine the cited documents and the requisite expectation of success is found by inference from a reading of Zhang and Lee.

Submitted herewith as Exhibit 1 (and in the accompanying Supplemental IDS filed concurrently herewith) is the full text of Lee. As set forth above, Lee explicitly discloses that reversed phase HPLC is the ***only*** technique by which, *e.g.* vitamin D_3 can be resolved. (Page 482, Col. 2 and Figure 5). Lee attributes this ability to separate, *e.g.* vitamin D_3, to the "reversed phase conditions." (*Id.*).

In this regard, we note that Lee was published in 1995, and presumably had Zhang (which was published in 1990) before them. Accordingly, contrary to the rejection's unsupported assertion that one of skill would "infer" from Zhang and Lee that the documents are properly combinable, Lee, published five years after Zhang, specifically and unambiguously comes to the opposite conclusion advanced by the rejection - that normal HPLC and reversed phase HPLC systems are ***not*** interchangeable for the separation of *e.g.* vitamin D_3.

It is well settled that it is improper to combine references where the references teach away from their combination. *See* MPEP §2145 at 2100-123 and *In re Grasselli*, 218 USPQ 769, 779 (Fed. Cir. 1983). Here, Lee specifically discloses that the reversed phase system succeeded in resolving vitamin D_3 whereas both normal phased and supercritical fluid HPLC failed. (Page 482, Col. 2). Thus, the "teachings" of Zhang and Lee clearly conflict. Accordingly, the Examiner had the burden to determine how one of skill in this art would resolve such a

conflict. *See* MPEP §2143.01 at 2100-98 ("Where the teachings of two or more prior art references conflict, the Examiner ***must*** weigh the power of each reference to suggest solutions to one of skill in the art ...."). This the rejection has not done. Thus for this additional reason, the rejection should be withdrawn.

A *prima facie* case of obviousness requires that the rejection describe with specificity ***why*** one skilled in the art would have combined the references to arrive at the claimed invention. *In re Dembiczak*, 50 USPQ2d 1614, 1617 (CAFC 1999). ("Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of *the requirement for a showing of the teaching or motivation to combine prior art references.*"). *See also In re Kotzab*, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000). ("[A] rejection cannot be predicated on the mere identification ... of individual components of claimed limitations. Rather, ***particular findings must be made*** as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed.")

Here, the rejection contains no such explanation or particular findings of fact. At most, the rejection relies on a nebulous "infer" standard that completely ignores Lee's teaching away without providing a scintilla of evidence to explain why one skilled in this art would ignore the express disclosure of Lee that only a reversed phase HPLC process would separate, *e.g.* vitamin D_3. But this too was the Examiner's burden. Rather, in the present case, the rejection uses the present specification as a blue print to interpret the disparate disclosures of Zhang and Lee, and in doing so has fallen into the same "hindsight trap" as the Examiner and Board did in *Kotzab*. (*Id.* at 1318). For this additional reason, the rejection should be withdrawn.

In sum, because the rejection provides no evidence or reasoning to explain why one should ignore the express teaching of Lee, the rejection is left with the acknowledged gap (*i.e.*, no teaching of a liquid or supercritical CO_2 for the mobile phase) that it cannot fill based on the evidence and reasoning set forth in the rejection.

Accordingly, for the reasons set forth above, entry of the amendments, withdrawal of the rejections, and allowance of the claims is respectfully requested. If the Examiner has any questions regarding this paper, please contact the undersigned.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, on October 3, 2000.

Kevin C. Hooper

Respectfully submitted,

By: _____

Kevin C. Hooper
Registration No. 40,402
BRYAN CAVE LLP
245 Park Avenue
New York, NY 10167-0034
Phone: (212) 692-1800
Fax: (212) 692-1900

EXHIBIT 1

Applications of Reversed-Phase High Performance Liquid Chromatography Using Enhanced-Fluidity Liquid Mobile Phases

Stephen T. Lee and Susan V. Olesik*
Department of Chemistry, The Ohio State University, 120 W. 18th Ave., Columbus, Ohio 43210, USA

Steven M. Fields
Marion Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215, USA

Abstract. Enhanced-fluidity liquid mobile phases (methanol/H_2O/CO_2) were used as eluents in reversed-phase HPLC. The low pressure drop across the column allowed serial connection of micro-scale columns to achieve the efficient separation of a coal tar sample. Other applications such as the separation of fat soluble vitamins and probucol and related compounds are shown.

Key words: *HPLC, enhanced-fluidity, viscosity, pressure drop*

INTRODUCTION

Supercritical fluids have markedly lower viscosities than those of liquids. For example, the viscosity of supercritical CO_2 at 40°C and 136 atm is 0.065 cP [1] compared to 0.35, 0.56, and 0.89 cP for acetonitrile, methanol, and water, respectively, at 25°C and ambient pressure. The low viscosities of supercritical fluid mobile phases result in increased solute diffusion, faster speed of analysis, and lower pressure drops across the chromatographic column in SFC than in HPLC. The low column pressure drop has facilitated the use of long and/or coupled columns in packed-column SFC to increase the total number of theoretical plates for separation of complex mixtures [2–4].

We have demonstrated the use of enhanced-fluidity (or low viscosity) liquid mobile phases as eluents in reversed-phase HPLC [5, 6]. We defined an enhanced-fluidity mobile phase as a common HPLC eluent to which high proportions of a low viscosity liquid, such as CO_2, has been added [7]. The previous publications demonstrated increased speed-of-analysis, chromatographic efficiency and decreased pressure drop across the chromatographic column in HPLC when enhanced-fluidity eluents were used. Herein, we demonstrate that the low pressure drop caused by the use of an enhanced-fluidity mobile phase can allow ready serial coupling of columns. These coupled columns were applied to the separation of a complex mixture—a coal tar standard. We also show additional applications such as the separation of fat-soluble vitamins and the separation of pharmaceuticals, such as probucol, and some closely related compounds with an enhanced-fluid mobile phase.

MATERIALS AND METHODS

The methanol/H_2O/CO_2 mixtures were prepared using two ISCO LC-2600 high-pressure syringe pumps. A known volume of a methanol/H_2O mixture at a composition of 0.70/0.30 mole fraction was placed in one pump. Liquid CO_2 at 136 atm and ambient temperature was held in another pump. Using the known density of CO_2 at these conditions, the appropriate volume of CO_2 was delivered to the pump holding the methanol/H_2O mixture. The methanol/H_2O/CO_2 mixture was then pressurized to 204 atm and allowed to equilibrate at ambient temperature for at least 12 h to ensure complete mixing of the solution.

*To whom correspondence should be addressed.

 CCC 1040-7685/95/05477-07

All experiments described in this article were performed using a 0.70/0.30 mole fraction methanol/H_2O mixture or a 0.49/0.21/0.30 mole fraction methanol/H_2O/CO_2 mixture. For convenience, the methanol/H_2O mixtures at 26 and 60°C are designated as the RT (room temperature) and ET (elevated temperature) mobile phases, respectively. The 0.49/0.21/0.30 mixtures at 26 and 60°C are designated as EF (enhanced-fluidity) and ET-EF (elevated temperature—enhanced-fluidity) mobile phases, respectively.

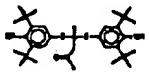
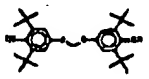
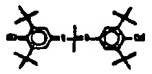
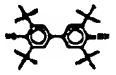
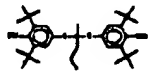
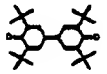
The chromatographic system consisted of an ISCO LC-2600 syringe pump (ISCO, Lincoln, NE), a Valco W-series high pressure injection valve with an injection volume of 200-nL (Valco Instruments, Houston, TX), BDS Hypersil C18, 150-mm × 1-mm columns packed with 5-μm diameter particles (Keystone Scientific, Bellefonte, PA) and a Spectra-Physics UV2000 UV/vis absorbance detector equipped with a capillary flow cell (model 9550-0155). The oven was that of a Carlo Erba Fractovap 4160 gas chromatograph. The mobile phase was preheated for the elevated temperature experiments by placing a 2-m length of 1/16-in i.d. stainless steel tubing inside the oven, after the syringe pump, and prior to the injector. The flow cell for detection was created by removing the polyimide coating from a 5-mm section of 100-μm i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ) and centering it in the capillary flow cell. The detector excitation wavelength was 254 nm for the polycyclic aromatic hydrocarbon test mixes and 230 nm for the probucol and vitamin test mixes. An Omega model PX931-5KSV pressure transducer (Omega Engineering, Stamford, CT) was placed in-line after the detector and before a post-detection restrictor. The flow control for the chromatographic system was maintained by a post detection restrictor that was an appropriate length of 20, 15, or 10-μm i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ). The outlet pressure of the column was monitored because the column pressure must be maintained above a minimum p to prevent the methanol/H_2O/CO_2 mixture from separating into two phases (liquid-gas). For example, a 0.506/0.218/0.276 mole fraction methanol/H_2O/CO_2 mixture at 59.7°C separates into two phases at pressures lower than 108.2 atm [8]. All experiments in this study were performed under conditions in which the methanol/H_2O/CO_2 mixture was a single liquid phase. The column inlet pressure was maintained at 204 atm throughout the chromatographic experiments except when using the RT mobile phaser with 4 columns in series. Under the RT conditions, a column inlet pressure of 320 atm was required to obtain the appropriate mobile phase linear velocity.

The primary chromatographic test mixture used in this study was a methanol solution containing 0.71 μL/mL benzene, 0.079 mg/mL naphthalene, 0.021 mg/mL anthracene, 0.082 mg/mL pyrene, 0.036 mg/mL benz[a]anthracene, 0.071 mg/mL benzo[e]pyrene, 0.039 mg/mL benzo[a]pyrene, and 0.046 mg/mL benzo[ghi]perylene. A Supelco test mix of 16 polyaromatic hydrocarbon compounds was also used (catalog #4-8905). This test mix was received in a methylene chloride/benzene solvent which was evaporated to dryness and replaced with methanol. The final concentration of each individual component of this test mix was 0.030 mg/mL.

A standard test mixture of vitamins was a methanol solution containing 0.02 mg/mL trans-retinol (vitamin A), 0.14 mg/mL butylated hydroxytoluene (BHT), 0.21 mg/mL trans-retinal (vitamin A aldehyde), 0.15 mg/mL ergocalciferol (vitamin D_2), 0.16 mg/mL cholecalciferol (vitamin D_3), 0.17 mg/mL retinol acetate (vitamin A acetate), 0.13 mg/mL ± α-tocopherol (vitamin E), 0.10 mg/mL ± α-tocopherol acetate (vitamin E acetate), and 0.12 mg/mL vitamin K_1. All vitamin standards were purchased from Sigma Chemical Company (St. Louis, MO) with the exception of retinol which was purchased from Aldrich (Milwaukee, WI). A mixture of probucol and related analogues was also characterized. The test mixture of these was a methanol solution containing 0.12 mg/mL compound 1, 0.084 mg/mL compound 2, 0.11 mg/mL probucol, 0.10 mg/mL compound 4, 0.096 mg/mL compound 5, and 0.11 mg/mL compound 6. The compound number and structures are shown in Table I. The probucol and analogue standards were donated by Marion Merrell Dow, Inc. (Cincinnati, OH). SRM 1597, a complex mixture of PAH isolated from coal tar, was obtained from The National Institute of Standards and Technology (Gaithersburg, MD).

Data were collected on an IBM AT-compatible computer using a data collection program written in our lab with ASYST 2.1 (Macmillan Software Company, New York, NY). Theoretical plates were determined by fitting a

Table I. *Number and structure of compounds in the probucol and analogue standard.*

Compound	Structure
1	
2	
3 Probucol	
4	
5	
6	

Gaussian peak to the experimental data using PeakFit 3.0 (PeakFit Analysis Software, Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

Injection Profile. Methanol was chosen as the injection solvent because it is the main constituent of the mobile phase and all of the test compounds were readily soluble in it. An extra peak was always observed in the chromatograms. This peak was also a negative deflection from the baseline. This type of "system peak" is expected when a mixed mobile phase is used and the injection solvent is not the same composition as the eluent [9]. Therefore, with methanol as the injection solvent and methanol/H_2O/CO_2 mixtures as the eluents, system peaks would be expected. As long as the system peak and the analyte peaks do not overlap, the system peak does not effect the retention of the analytes under investigation. To test whether the negative peak was a system peak, the methanol/H_2O/CO_2 mixture was used as an injection solvent for one study.

The sample was injected with the pressurized methanol/H_2O/CO_2 mobile phase by splitting mobile phase eluting from the pump with a three-way valve. The majority of the mobile phase was delivered through the tubing, injector, column, detector, restrictor in that order as usual. The remaining mobile phase split at the three-way valve was delivered to a 1.2 mL extraction cell containing the sample solutes. The sample to be injected was prepared by delivering 1.2 mL of the chromatographic test mix to the extraction cell with a glass pipet and evaporating the methanol sample solvent with a stream of nitrogen. This process was repeated once so that the total volume of test mix delivered to the cell and evaporated was ca. 2.4 mL. The cell containing the sample solutes was then put in line between the three-way valve and the injector. The mobile phase solvated and pressurized the contents of the extraction cell. The cell and sample loop were maintained at the head pressure of the system by a valve positioned after the injector. A restrictor directly after the valve was created with a length of 5 μm id fused silica. By opening the post injector valve, sample was allowed to flow from the extraction cell and fill the sample loop. Using this system, triplicate injections of the 16-component standard PAH mixture were made. This was compared to triplicate injections of the 16-component PAH standard mixture using a conventional syringe guide and waste line respectively.

Figure 1 shows chromatograms using methanol as the injection solvent and using the 0.49/0.21/0.30 mole fraction methanol/H_2O/CO_2 mobile phase as the injection solvent. This comparison shows no difference in terms of efficiency (plate height) or selectivity (k') for the separation of the 16-component PAH test mix. However, the phase non-equilibrium introduced by using methanol as the injection solvent is shown in Figure 1A. A large positive deflection followed by a large negative deflection in the signal was observed when using methanol as the injection solvent. Only a small negative deflection in the baseline when using the mobile phase as the sample solvent is observed. The small negative deflection is attributed to methanol that was not completely evaporated under the nitrogen stream. In addition, some of the more volatile low molecular weight components (benzene, naphthalene, acenaphthalene, and fluoranthene) were lost when the methanol was evaporated under the nitrogen stream. Because there was no observable effect on the chromatographic performance when using methanol as the injection solvent, besides the observed system peak, methanol was used as the injection solvent for all other experiments in this study.

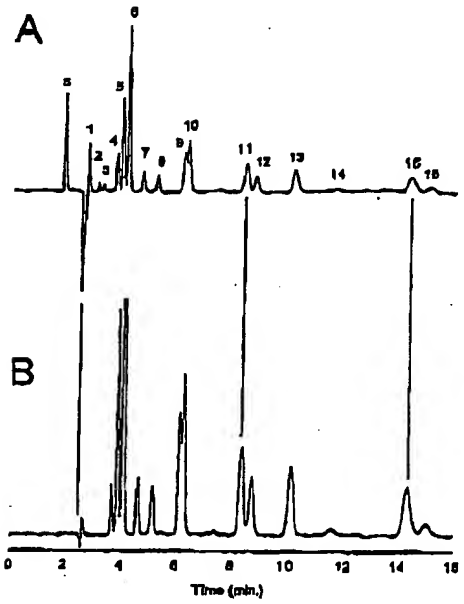


Figure 1. *Chromatograms with the EF mobile phase at 204 atm using (A) methanol and (B) 0.49 / 0.21 / 0.30 mole fraction methanol / H_2O / CO_2 mixture (EF mixture) as the injection solvent. (s) solvent, (1) benzene, (2) naphthalene, (3) acenaphthalene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluorene, (8) pyrene, (9) benz [a]anthracene, (10) chrysene, (11) benzo[b] fluoranthene, (12) benzo[k]fluoranthene, (13) benzo[a]pyrene, (14) dibenzo[a,h]anthracene, (15) indeno[1,2,3-cd]pyrene, (16) benzo[ghi]perylene.*

Pressure drop. Table II shows the pressure drop across the chromatographic system and the average number of theoretical plates for peaks 3–7 in Figure 1 at the 4 mobile phase conditions defined previously. The data were obtained at approximately the same linear velocity for 1 column and 4 columns in series. The data in Table I show the pressure drop across 4 chromatographic columns in series is roughly four-fold the pressure drop of one column for each of the 4 mobile phase conditions studied. The pressure drop across the column decreases in the following order of mobile phase conditions: methanol/H_2O at room temperature > methanol/H_2O at elevated temperature > enhanced-fluidity mixture at room temperature > enhanced-fluidity mixture at elevated temperature. Darcy's law [10, 11] describes the relationship between pressure drop, ΔP, column length, L, mobile phase viscosity, η, average linear velocity, $\langle u \rangle$, and particle diameter, d_p, in porous beds, such as chromatographic columns:

$$\Delta P = \frac{\phi \eta \langle u \rangle L}{d_p^2}$$

where ϕ is the dimensionless flow resistance parameter which typically has values in the range of 500–1000. The data in Table I are consistent with Darcy's law in that a linear increase in pressure drop with column length is predicted and observed. This expression and the data in Table I show the diminished pressure drop obtained by elevating the temperature of the methanol/H_2O mobile phase from 26–60°C, or by adding CO_2 to the mixture.

When the RT mobile phase with 4 columns at a linear velocity of 0.189 cm/s was used, the observed pressure drop across the chromatographic system was 314.2 atm (4617 psi). This pressure approaches the maximum pressure limits for many HPLC components currently in use. For example, the Valco injection valve

Table II. *Variation in column pressure drop and efficiency with mobile phase conditions and column length.*

One column	Mobile phase condition	Linear velocity (cm/s)	ΔP (atm)	N_{3-7} (plates)	N_{3-7} (plates/m)
	RT	0.196	81.3	7,618	50,787
	ET	0.204	49.2	8,880	59,200
	EF	0.207	39.2	9,955	66,367
	ET-EF	0.200	24.4	11,068	73,787
Four columns					
	RT	0.189	314.2	30,290	50,483
	ET	0.199	148.7	40,262	67,103
	EF	0.189	144.8	44,124	73,540
	ET-EF	0.182	81.9	47,948	79,913

used in this study has a maximum pressure rating of 340.2 atm (5000 psi). Therefore for the RT condition, the length of the chromatographic column cannot be extended significantly if one wishes to work at this linear velocity and conversely the linear velocity cannot be increased significantly using 4 columns in series.

Figure 2 compares a separation of PAH standards with the RT mobile phase on one column to that with the ET-EF mobile phase on 4 columns in series. The separations were both performed at approximately the same linear velocity, pressure drop, and analysis time. Retention is decreased with ET-EF mobile phase but the average number of theoretical plates for peaks 3–7 was more than six-fold greater for the ET-EF separation than for the RT separation. The dramatic increase in theoretical plate number can be attributed to several factors. The four-fold increase in column length accounts for ca. 64% of the total plate number for the ET-EF separation, leaving a 36% increase in plate number attributable to a combination of a shift in the optimum velocity to higher linear velocities, a decrease in the slope of the mass transfer region of the van Deemter curve, and decreased capacity factors (k'). See Reference 5 for a more detailed discussion of the relative contributions to plate height for the RT and ET-EF condition [5].

Figure 3 compares chromatograms of the coal tar (SRM 1597) sample separated at the same linear velocity with the EF mobile phase on 1 and on 4 columns. This comparison illustrates the improvement in efficiency when using 4 columns in series compared to one. However, at the same linear velocity, the analysis time is 4 times as long with 4 columns. The peaks in Figure 3 were tentatively identified by injecting the Supelco test mix that contained 16 polyaromatic hydrocarbon compounds immediately after collecting the coal tar chromatogram and superimposing these chromatograms on one another. In addition to the 13 compounds identi-

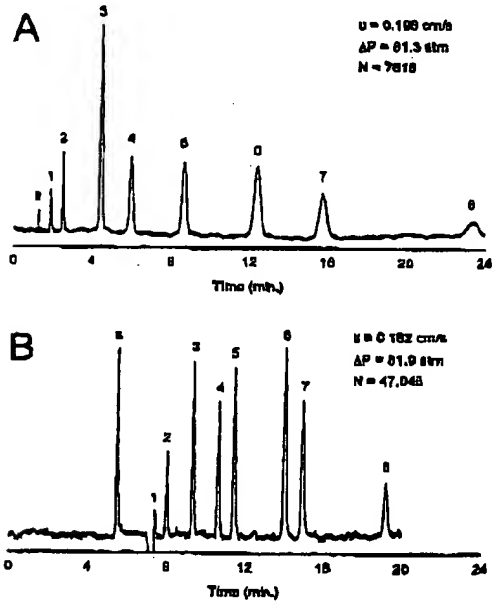


Figure 2. *Chromatograms at 204 atm with approximately the same column pressure drop but with (A) the RT mobile phase and 1 column; and (B) the ET-EF mobile phase and 4 columns. (s) solvent, (1) benzene, (2) naphthalene, (3) anthracene, (4) pyrene; (5) benz[a]anthracene, (6) benzo[e]pyrene, (7) benzo[a]pyrene, (8) benzo [ghi]perylene.*

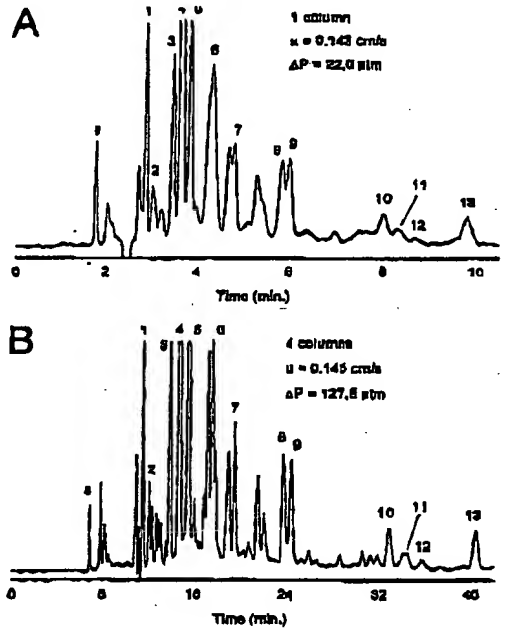


Figure 3. *Chromatograms of SRM 1597 coal tar standard using the EF mobile phase at 204 atm with (A) 1 column and (B) 4 columns. (s) solvent, (1) naphthalene, (2) acenaphthalene, (3) fluorene, (4) phenanthrene, (5) anthracene, (6) fluoranthene, (7) pyrene, (8) benz[a]anthracene, (9) chrysene, (10) benzo[b]fluoranthene, (11) benzo[k]fluoranthene, (12) perylene, (13) benzo[a]pyrene.*

fied in Figure 3, we identified 2 additional solutes, indeno[1,2,3-cd]pyrene and benzo[ghi]-perylene, at longer retention times; these peaks are not shown because the chromatograms were truncated to illustrate the increase in efficiency using the 4 columns in series. The identifications were further supported by referring to an article by Wise et al. and correlating our results with this extensive LC, GC, GC/MS analysis of the same sample [12].

APPLICATIONS

Probucol is a fat-soluble antioxidant that has been shown to lower serum cholesterol concentrations in humans [13]. The separation of some structural analogues of probucol were also investigated. Satonin and Coutant developed GC and HPLC methods for the analysis of the probucol and analogues [14]. The HPLC method was preferred because probucol decomposition occurred in GC analyses unless careful temperature control was used in the separation. The HPLC method of Satonin et al. was a reversed-phase separation using acetonitrile/hexane/0.1M ammonium acetate. As mentioned in previous sections of this paper, and demonstrated elsewhere [5], enhanced-fluidity liquids as eluents allow increased speed of analysis or increased efficiency due to the increased diffusion rates in the mobile phase. Therefore, a separation of compounds that would otherwise only be accomplished by elevated temperature HPLC can be accomplished by enhanced-fluidity LC at room temperature. Figure 4 is a separation of probucol and 5 structurally related compounds using EF mobile phase conditions across one column. The structures of the labeled compounds are listed in Table I. Baseline resolution was achieved for all compounds with the exception of compounds 2 and 3 in under 11 min. By contrast, peak 4 elutes at 43 min and peak 5 elutes at 75 min under RT conditions at the same linear velocity.

HPLC is the most commonly used technique for the analysis of fat-soluble vitamins. Several in-depth reviews on the chromatographic analysis of vitamins are available [15–17]. HPLC in both the reversed-phase and normal-phase modes is used for the analysis of fat-soluble vitamins. Packed column [18–20] and open tubular [21] supercritical fluid chromatography have also been investigated for the analysis of fat soluble vitamins. Reversed-phase HPLC is frequently used when simultaneous

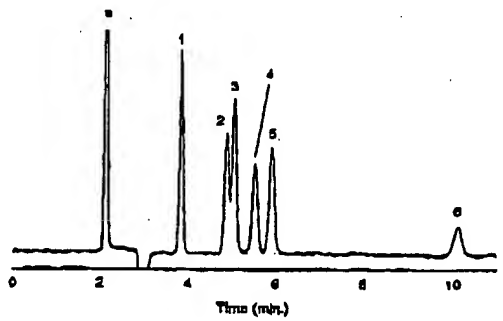


Figure 4. *Chromatogram of probucol and related compounds using the EF mobile phase at 204 atm.*

determination of several different vitamins in one chromatographic run is desired. Reversed-phase HPLC, unlike normal phase HPLC and SFC, is the only technique by which vitamins D_2 (ergocalciferol), and D_3 (cholecalciferol) can be resolved. Figure 5 shows a separation of fat-soluble vitamins with the EF mobile phase conditions across one column. Baseline resolution for most of these compounds is achieved in under 20 min. Partial resolution of vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) is also achieved. This is attributed to the separation taking place under reversed-phase conditions.

In summary, EF and ET-EF mobile phases are low viscosity mobile phases that are viable choices for extending the length of the chromatographic column to achieve a higher total number of theoretical plates for separations. These low viscosity mobile phases have previously been shown to increase the optimum lin-

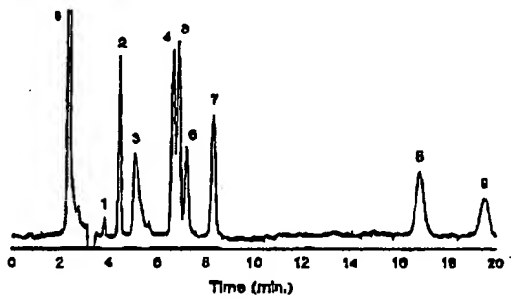


Figure 5. *Chromatogram of fat soluble vitamins using the EF mobile phase at 204 atm. (s) solvent, (1) trans-retinol, (2) BHT, (3) trans-retinal, (4) ergocalciferol, (5) cholecalciferol, (6) retinol acetate, (7) ±-tocopherol, (8) ±-tocopherol acetate, (9) vitamin K_1.*

ear velocity of the mobile phase and extend the operable linear velocity range resulting in shorter analysis times. In addition, the separations shown in this article demonstrate the applicability of enhanced-fluidity mobile phases in reversed-phase HPLC for a range of different types of compounds.

ACKNOWLEDGMENTS

The authors thank Keystone Scientific for providing the chromatographic columns for this work. We thank Stephen Wise of NIST for providing the SRM coal tar standard. This work was supported by the National Science Foundation under Grant CHE-9118913.

REFERENCES

1. K. Stephan and K. Lucas, *Viscosity of Dense Fluids* (Plenum, New York, 1979), p. 77.
2. A. Malik, W. Li, and M.L. Lee, *J. Microcol. Sep.* 5, 361 (1993).
3. T.A. Berger and W.H. Wilson, *Anal. Chem.* 65, 1451 (1993).
4. P. Sandra, A. Kot, and F. David, *Proceedings of the 16th International Symposium on Capillary Chromatography*, Riva del Garda, Italy, Sept. 27–30, 1994, p. 1515.
5. S.T. Lee and S.V. Olesik, *Anal. Chem.* 66, 4498 (1994).
6. Y. Cui and S.V. Olesik, *J. Chromatogr.* 691, 151 (1995).
7. Y. Cui and S.V. Olesik, *Anal. Chem.* 63, 1812 (1991).
8. S.T. Lee and S.V. Olesik, unpublished data.
9. S. Levin and E. Gruska, *Anal. Chem.* 58, 1602 (1986).
10. A.E. Scheidegger, *The Physics of Flow Through Porous Media* (Macmillan, New York, 1960), p. 68.
11. J.C. Giddings, *Dynamics of Chromatography, Part I, Principles and Theory* (Marcel Dekker, New York, 1965).
12. S.A. Wise, B.A. Benner, G.D. Byrd, S.N. Chesler, R.E. Rebbert, and M.M. Schantz, *Anal. Chem.* 60, 887 (1988).
13. S.J.T. Mao, M.T. Yates, A.E. Rechtin, R.L. Jackson, and W.A. Van Sickle, *J. Med. Chem.* 34, 298 (1991).
14. D.K. Satonin and J.E. Coutant, *J. Chromatogr.* 380, 401 (1986).
15. A. Rizzolo and S. Polesello, *J. Chromatogr.* 624, 103 (1992).
16. G.F.M. Ball, *J. Micronutr. Anal.* 4, 255 (1988).
17. A.P. De Leenheer, H.J. Nelis, W.E. Lambert, and R.M. Bauwens, *J. Chromatogr.* 429, 3 (1988).
18. K. Matsumoto, S. Tsuge, and Y. Hirata, *Chromatographia* 21, 617 (1986).
19. K. Jinno, *SFC Applications*, K.E. Markides and M.L. Lee, Eds. (Brigham Young University Press, Provo, UT, 1988).
20. Y. Hirata, *SFC Applications*, K.E. Markides and M.L. Lee, Eds. (Brigham Young University Press, Provo, UT, 1988).
21. C.M. White, D.R. Gere, D. Boyer, P. Pacholec, and L.K. Wong, *J. High Resolut. Chromatogr./ Chromatogr. Commun.* 11, 94 (1988).

Received: May 1, 1995
Accepted: July 19, 1995